

## REVIEWS

## Therapeutic potential of Toll-like receptor 9 activation

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**Abstract** | In the decade since the discovery that mouse B cells respond to certain unmethylated CpG dinucleotides in bacterial DNA, a specific receptor for these 'CpG motifs' has been identified, Toll-like receptor 9 (TLR9), and a new approach to immunotherapy has moved into the clinic based on the use of synthetic oligodeoxynucleotides (ODN) as TLR9 agonists. This review highlights the current understanding of the mechanism of action of these CpG ODN, and provides an overview of the preclinical data and early human clinical trial results using these drugs to improve vaccines and treat cancer, infectious disease and allergy/asthma.

### Pattern-recognition receptors

Receptors that bind to molecular patterns found in pathogens but not mammalian cells. Examples include the mannose receptor, which binds to terminally mannosylated and polymannosylated compounds, and Toll-like receptors, which are activated by various microbial products, such as bacterial lipopolysaccharides, hypomethylated DNA, flagellin and double-stranded RNA.

### CpG motifs

DNA oligodeoxynucleotide sequences that include an unmethylated cytosine-guanosine sequence and certain flanking nucleotides, which have been found to induce innate immune responses through interaction with the Toll-like receptor 9.

Vertebrates are endowed with two complementary immune systems, the innate and the adaptive. The adaptive immune system is mediated by the highly sophisticated and recently evolved B and T cells, which specifically target the invader, and provide a memory response to prevent a repeat of the infection. The innate immune system, in contrast to the adaptive system, was relatively neglected for many decades until recent discoveries provided a remarkable new understanding of how it accomplishes its crucial mission. To protect the host from succumbing to infections, the innate immune system, which is evolutionarily more ancient than adaptive immunity, must accomplish four fundamental tasks (BOX 1). First, it must rapidly detect any infectious agent, regardless of whether it is a virus, bacteria, fungus or parasite. Second, innate immune cells seem to rapidly categorize the type of invading infectious agent as to whether it is located extracellularly or intracellularly. Third, innate immune defences appropriate to the pathogen class are activated to either eradicate or at least temporarily contain the infection. Fourth, innate immune cells induce the appropriate type of adaptive immune response to eliminate the infection and prevent its recurrence.

The key feature of innate immune cells that enables them to detect and categorize infection seems to be their repertoire of what have been termed pattern-recognition receptors (PRRs), which bind certain general types of molecules that are expressed across broad classes of pathogens, but which are absent or restricted in some way in vertebrates. The best understood family of PRRs are the Toll-like receptors (TLRs), of which 10 are known in humans (reviewed in REF. 1). TLRs that are specific for molecules characteristic of extracellular pathogens, such

as lipopolysaccharides or lipopeptides, are expressed at the cell surface, whereas TLRs that detect intracellular pathogens are expressed within innate immune cells and are specific for nucleic acids. For example, TLR9 detects unmethylated CpG dinucleotides, which are relatively common in the genomes of most bacteria and DNA viruses, but which are suppressed and methylated in vertebrate genomes. The endosomal localization of TLR9 allows efficient detection of invading viral nucleic acids, while preventing 'accidental' stimulation by CpG motifs within self DNA<sup>2</sup>. Although beyond the scope of this review, it should be noted that a TLR9-independent cytosolic pathway of DNA detection has recently been demonstrated, perhaps indicating the importance of this capability for innate immunity<sup>3,4</sup>.

Different immune cells express distinct subsets of the TLRs, which likely enables the immune system to tailor its responses against different pathogen classes<sup>1</sup>. Among resting human immune cells TLR9 is expressed primarily or exclusively in B cells and plasmacytoid dendritic cells (pDC), a specialized type of dendritic cell that produces most of the type I interferons (IFN) that are made in response to viral and intracellular pathogens<sup>5</sup> (reviewed in REF. 1). Some studies have also reported functional TLR9 expression in activated but not in resting human neutrophils<sup>6</sup> and pulmonary epithelial cells<sup>7,8</sup>, but the biological significance of this TLR9 expression is uncertain.

Unfortunately for immunologists, the cellular patterns of TLR expression vary between different species. For example, mice differ from primates in that they express TLR9 not only in pDC and B cells, but also in monocytes and myeloid dendritic cells (reviewed in REF. 1).

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## Box 1 | Roles of innate immunity

### Detection

Invading pathogens are detected by one or more members of several general families of 'pattern-recognition receptors' (PRRs), which include:

- TLRs (Toll-like receptors): 10 members known in humans, with diverse ligands
- NOD (nucleotide-binding oligomerization domain): detect muramyl dipeptide of peptidoglycan
- RIG1 (retinoic acid-inducible protein 1)-related proteins: detect dsRNA
- Mannose receptor: detects mannosylated lipoarabinomannans
- C-type lectins such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin): detect various antigens

PRRs detect molecules that have been called pathogen-associated molecular patterns (PAMPs); however, this term is slightly misleading, as none of these molecules are actually restricted in their expression to pathogens. Instead, what seems to distinguish a pathogen from a commensal organism is the anatomic or intracellular location of the molecule. For example, molecules from commensal flora would not be expected to stimulate a PRR on a basolateral epithelial surface, or to reach an intracellular PRR.

### Categorization

Extracellular pathogens can be 'recognized' by cell-surface PRRs that bind broadly conserved structures such as flagellin (a highly conserved protein needed by motile organisms) and lipopolysaccharides. By contrast, detection of intracellular pathogens seems to be accomplished by intracellular receptors, including certain of the TLRs that are intra-endosomal, and RIG-I, which is cytoplasmic.

### Containment

Depending on the type of infection, distinct subsets of innate immune cells produce cytokines and chemokines appropriate to limiting the spread of the infection. The response to an extracellular pathogen is typically dominated by pro-inflammatory cytokines such as tumour-necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-12, whereas in the case of an intracellular pathogen the crucial innate immune products for control are the type I interferons.

### Elimination and memory

The innate immune system can sometimes eradicate the infection on its own, in which case there will be no immune 'memory' of the event. Immune memory resides in adaptive immune cells, including both a humoral arm (B cells, which produce antibodies that can kill extracellular pathogens and prevent infection by intracellular pathogens) and a cellular arm (killer T cells, which are the most efficient killers of cells infected by intracellular pathogens).

This makes it difficult at best to predict accurately the effects of TLR9 activation in humans by extrapolating from results obtained in mice, in which more types of immune cells are activated by TLR9 agonists. This review will focus on the mechanisms and therapeutic applications of activating TLR9 with synthetic CpG oligodeoxynucleotide (ODN) agonists, which are currently in human clinical trials in the fields of infectious disease, cancer and asthma/allergy.

### Targeted immune activation by CpG ODN

Most types of immune cells do not express TLR9, and so are not activated directly by CpG DNA. All of the cellular immune effects of CpG ODN in humans are thought to result directly and indirectly from activating TLR9-expressing pDC and B cells (BOX 2). pDC activated through TLR9 secrete IFN $\alpha$ , which drives the migration and clustering of pDC in the marginal zone and outer T-cell areas of the lymph node, where they are better able to stimulate adaptive immune responses<sup>9</sup>. TLR9-stimulated B cells and pDC show increased expression of co-stimulatory molecules, resistance to apoptosis,

upregulation of the chemokine (C-C motif) receptor CCR7, and secretion of T<sub>H</sub>1-promoting chemokines and cytokines such as monocyte inflammatory protein-1 (MIP1), IFN $\gamma$ -inducible 10-kDa protein (IP10) and other IFN-inducible genes<sup>10</sup>. Co-activation of naive, germinal centre or memory B cells through the B-cell-antigen receptor and TLR9 induces their differentiation into plasma cells<sup>11</sup>; for memory B cells, activation through TLR9 alone is sufficient to drive differentiation to plasma cells<sup>12,13</sup>. The B-cell-stimulatory effect of TLR9 is so great that the efficiency of hybridoma generation from purified primary human memory B cells is improved from 1–2% without CpG to 30–100% with the addition of a CpG ODN<sup>14</sup>. Although CpG-induced plasma cell differentiation does not require T-cell help, its efficiency is enhanced by interactions with pDC, together with B-cell receptor crosslinking<sup>15</sup>. The net effect of TLR9 activation is to induce T<sub>H</sub>1-biased cellular and humoral effector functions of innate and adaptive immunity (TABLE 1).

Even before the discovery that TLR9 was an intracellular protein, it was apparent that stimulation of immune cells by CpG ODN requires internalization<sup>16</sup>. ODN internalization occurs spontaneously in culture without the need for uptake enhancers or transfection, is temperature- and energy-dependent, and seems to be relatively sequence-independent. The earliest steps in the CpG-induced signal transduction pathways can be blocked by inhibitors of endosomal acidification/maturation<sup>17–20</sup> or by inhibitors of phosphatidylinositol 3-kinase, which seems to have a role in ODN internalization<sup>21</sup>. Following internalization into an endosomal compartment, CpG motifs seem to be bound and recognized by TLR9, leading to the rapid recruitment and/or activation of the adaptor molecules MyD88, interleukin-1 receptor-associated kinase-1 (IRAK1), interferon regulatory factor-7 (IRF7), and tumour-necrosis factor- $\alpha$  receptor activated factor-6 (TRAF6)<sup>18,22–26</sup>. This results in the rapid activation of several mitogen-activated protein kinases, including extracellular receptor kinase (ERK), p38, and Jun N-terminal kinase, as well as the I $\kappa$ B complex, and these pathways converge on the nucleus to alter gene transcription<sup>27–33</sup>.

### Structure–activity relationships of CpG ODN

The two bases to the 5' and 3' sides of the CpG dinucleotide comprise a CpG motif, one of which is sufficient for immune stimulation<sup>16</sup>. Early empirical structure–activity relationship (SAR) studies revealed species-specific differences in the optimal CpG motif, which is GACGTT for mice<sup>16,34,35</sup> but GTCGTT for humans<sup>27</sup>. Once TLR9 was identified as the CpG receptor, it was possible to show that the species-specific recognition was present at the level of the TLR9 itself<sup>6</sup> and that TLR9 directly binds DNA<sup>26,37</sup>.

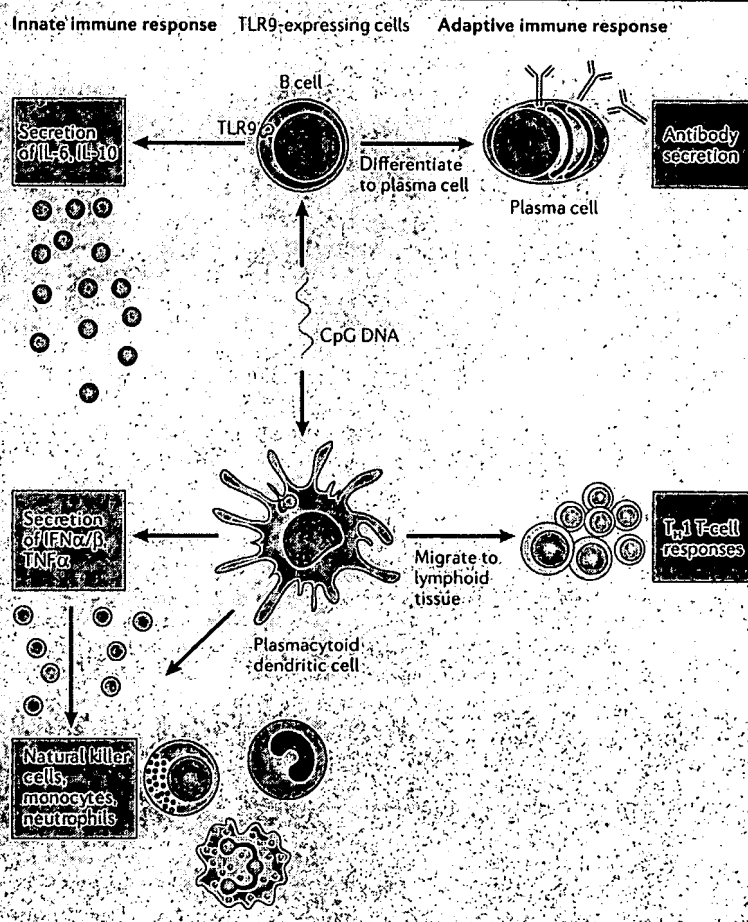
Besides the hexamer CpG motif, the immune-stimulatory activity of an ODN is determined by the number of CpG motifs it contains (usually two to four are optimal), the spacing of the CpG motifs (usually at least two intervening bases, preferably thymine residues, is optimal), the presence of poly-G sequences or other flanking sequences in the ODN (effect depends on

**Plasmacytoid dendritic cell (pDC).** A unique type of dendritic cell. These cells are also known as interferon (IFN)-producing cells because they are the main source of type I IFNs (such as IFN $\alpha$  and IFN $\beta$ ) during viral infections.

**Co-stimulatory molecules.** Soluble or membrane-bound molecules that signal to T cells (or other immune cells) and, having little or no effect alone, either enhance or modify the physiological effect of the primary signal, which is mediated by engagement of the T-cell receptor (or other receptors on other immune cells).

**Box 2 | Role of TLR9 in triggering innate and adaptive immunity**

Two types of human immune cells express Toll-like receptor 9 (TLR9): B cells and plasmacytoid dendritic cells (pDC; see figure). Regardless of the presence or absence of CpG motifs, DNA is endocytosed into a cellular compartment where it is exposed to TLR9. If the DNA contains CpG motifs, then TLR9 is activated. In pDC, TLR9 activation is dependent on interleukin (IL)-1 receptor-associated kinase (IRAK)-4 and interferon regulatory factor-7 (IRF7), and requires direct interactions between IRF7 and MyD88, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) receptor activated factor-6 (TRAF6) and IRAK-1<sup>45-49</sup>. TLR9 activation induces nuclear factor- $\kappa$ B (NF- $\kappa$ B) and other intracellular signalling pathways that initiate a rapid innate immune response that is characterized by the secretion of a variety of proinflammatory and antiviral cytokines, such as IL-6, TNF $\alpha$  and type I interferons (IFN), and immune regulatory cytokines that limit the intensity of the inflammatory response, such as IL-10. There is also a reverse effect of CpG-activated B cells on dendritic cells, by which TLR9 activation drives CD5<sup>+</sup> B cells to produce IL-10, which then suppresses the T<sub>H</sub>1-priming function of the dendritic cells<sup>192</sup>. In contrast to some other innate immune responses, relatively little IL-12 is produced by TLR9-activated human immune cells. Natural killer (NK) cells and other innate immune cells are activated secondarily by pDC through both IFN-dependent and IFN-independent pathways. B cells activated through TLR9 have a greatly increased sensitivity to antigen stimulation, promoting their differentiation to antibody-secreting plasma cells, and therefore contributing to the adaptive immune response. TLR9-stimulated pDC migrate to the T-cell zones of lymph nodes and other secondary lymphoid tissues, express increased levels of co-stimulatory molecules that enhance their capacity to activate naive and memory T cells and have increased capacity to cross-present soluble protein antigens to CD8 T cells. As a consequence, CpG DNA promotes strong T<sub>H</sub>1, CD4 and CD8 T-cell responses.



ODN structure and backbone), and the ODN backbone (a nuclease-resistant phosphorothioate backbone is the most stable and best for activating B cells, but gives relatively weaker induction of IFN $\alpha$  secretion from pDC compared with native phosphodiester linkages in the CpG dinucleotide)<sup>16,27,35,38-41</sup>. In addition, the immune-stimulatory effects of the ODN are enhanced if there is a TpC dinucleotide on the 5' end and if the ODN is pyrimidine rich on the 3' side<sup>27,35,39,42</sup>.

For therapeutic applications CpG ODN are typically synthesized with at least partially phosphorothioate-modified (PS-ODN) backbones to provide nuclease resistance and increased half-life, and generally produce a greater immune-stimulatory effect. There are at least three classes of immune-stimulatory CpG ODN with distinct structural and biological characteristics (BOX 3). The capacity of the different CpG ODN classes to induce such divergent immune effects might seem surprising, because these effects all seem to result from the activation of a single receptor, TLR9<sup>43,44</sup>. Maximal induction of pDC IFN $\alpha$  secretion is associated with ODN that can form secondary structures, such as the dimeric C class and the multimeric A class. These higher-ordered structures might induce TLR9 crosslinking, promote the recruitment of one or more additional cofactors

or adaptor proteins into the TLR9 signalling complex, and/or alter the intracellular compartmentalization of the ODN<sup>45</sup>. It therefore seems that the biological activity of TLR9 can be modulated by designing ligands that bind it in different ways.

A wide range of DNA backbones, modifications and substitutions have been explored for their effects on the capacity of ODN to activate TLR9 and induce innate and adaptive immunity. These SAR studies have shown that such modifications can alter the magnitude and character of immune activation, within the range of effects shown for the different ODN classes described above (reviewed in REFS 46,47). TLR9 recognition of the CpG motif in a phosphorothioate backbone seems to be sensitive to the P chirality, with the R stereoisomer preferred<sup>48</sup>.

Several types of suppressive ODN (S-class ODN) have been shown to block TLR9 signalling, but do not block activation of immune cells through TLR4, CD40 or the B-cell receptor<sup>49-51</sup>. In contrast to original expectations, S-class ODN are not specific TLR9 inhibitors; some also block TLR7 and/or TLR8 and RNA-mediated immune activation, depending on the presence or absence of specific sequence motifs<sup>52-54</sup>. Although incompletely understood, the mechanism of suppression by S-class ODN is distal to ODN uptake, proximal to early signalling

Plasma cells  
Non-dividing, terminally  
differentiated  
immunoglobulin-secreting  
cells of the B-cell lineage.

Table 1 | Activation of both innate and adaptive humoral and cellular immunity by TLR9 agonists

| Immune system | Humoral effects   | Cellular effects   |
|---------------|---|--|
| Innate        | <ul style="list-style-type: none"> <li>• IFN<math>\alpha</math> secretion</li> <li>• Secretion of IFN-inducible chemokines and cytokines</li> <li>• Secretion of pro-inflammatory cytokines (IL-6, TNF<math>\alpha</math>)</li> <li>• Secretion of anti-inflammatory cytokines (IL-10, IL-1RA)</li> </ul> | <ul style="list-style-type: none"> <li>• NK cell lytic activity</li> <li>• Monocyte expression of TNF-related apoptosis-inducing ligand (TRAIL)<sup>198</sup></li> <li>• Neutrophil activation, migration and bacterial uptake<sup>70,199,200</sup></li> </ul> |
| Adaptive      | <ul style="list-style-type: none"> <li>• Induction of IgG isotype switching and antibody secretion</li> <li>• Suppression of IgE antibody production</li> </ul>   | <ul style="list-style-type: none"> <li>• Differentiation of T<sub>H</sub>1 cells</li> <li>• Enhanced cross-priming</li> <li>• Increased CTL</li> </ul>   |

CTL, cytotoxic T lymphocyte; IFN, interferon; IL-10, interleukin-10; Ig, immunoglobulin; IL-1RA, interleukin-1 receptor antagonist; NK, natural killer; TLR9, Toll-like receptor 9; TNF $\alpha$ , tumour-necrosis factor- $\alpha$ .

events such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation, and could involve direct blockade of the signal transduction cascade leading to interleukin-12 (IL-12) and IFN $\gamma$  production<sup>51,55</sup>. SAR studies have revealed that to most effectively block CpG-induced TLR9 activation, S-class ODN should contain a CCT motif near the 5' end, and at least one G-rich region three to five bases 3' to this<sup>56</sup>.

### Drug-like properties of synthetic CpG ODN

Some of the characteristics of synthetic ODN are quite attractive for drug development, whereas others are less favourable (BOX 4). The technology for commercial-scale (multi-kilogram) ODN synthesis and purification, carried out according to Good Manufacturing Practices, has been well developed during the past decade of antisense and aptamer drug development. Antisense and aptamer oligonucleotide drugs have been approved by the US FDA, establishing a regulatory pathway for this general class of drugs. The absorption, distribution, metabolism and elimination (ADME) properties of synthetic PS-ODN with and without CpG motifs have been well characterized and reported in the extensive literature on antisense ODN, which has shown these characteristics to be essentially sequence-independent<sup>57,58</sup>. ODNs given subcutaneously are slowly absorbed from injection sites (with the highest concentration in the draining lymph nodes for the first several days after injection), and then enter the systemic circulation, where they demonstrate high-capacity, low-affinity binding to plasma proteins, principally albumin. ODN are rapidly cleared into tissues, especially the liver, kidneys and spleen, but do not seem to cross the blood-brain or blood-testes barriers. Catabolism of ODN typically occurs by exonuclease digestion and base clipping, primarily at the 3' end, resulting in natural DNA bases and thiophosphate metabolites that are excreted in the urine. The immune effects of CpG ODN administration through different routes result from their ADME characteristics. For example, subcutaneous administration of CPG 7909 (Coley), which results in high levels of the compound in the draining lymph node (which would contain a relatively high concentration of TLR9-expressing cells), induces high levels of serum cytokines and chemokines<sup>59</sup>. On the other hand, even relatively high-dose intravenous administration of CPG 7909, which is rapidly diluted in the blood and is approximately 95% protein bound, fails to induce measurable serum cytokine responses in humans<sup>59</sup>. Because the

pharmacodynamics of subcutaneous CpG ODN result from the local ODN concentration in the draining lymph nodes, they do not match the systemic pharmacokinetics.

### Therapeutic applications of CpG ODN

**Prevention and therapy of infectious disease.** If the normal function of TLR9 is to stimulate protective immunity against intracellular pathogens, then it could be proposed that prophylactic or therapeutic treatment with a synthetic TLR9 ligand would provide protection against an intracellular infectious challenge and/or eliminate a chronic infection. Indeed, studies in mice have demonstrated that the innate immune defences activated by CpG ODN given by injection, inhalation or even by oral administration can protect against a wide range of viral, bacterial and even some parasitic pathogens, including lethal challenge with Category A agents or surrogates such as *Bacillus anthracis*, vaccinia virus, *Francisella tularensis* and Ebola virus<sup>60-77</sup>. The mechanisms of protection have only been partially investigated. Protection in a *Listeria monocytogenes* model has been linked to CpG-activated dendritic cells, which protect naive mice against adoptive transfer<sup>78-80</sup>. Additional cell types might also provide some protection, because naive mice that received CpG-pretreated spleen cells depleted of CD11c<sup>+</sup> dendritic cells still had a partial survival benefit. The parameters of protection in the different models vary widely — protection lasts for at least 2 weeks after a single CpG dose in models such as *L. monocytogenes* and *F. tularensis* (LVS strain)<sup>60,63,64</sup>, but only for a day or so in the vaginal herpes simplex virus challenge model<sup>81</sup>.

Post-exposure therapy with TLR9 activation is generally ineffective against rapidly progressive acute infectious agents. However, TLR9 activation rapidly suppresses hepatitis B virus (HBV) replication in transgenic mice<sup>82</sup>, suggesting potential utility of this approach in the treatment of chronic viral infections in humans. The antiviral effect in this model seemed to be indirect and secondary to CpG-induced IFN $\alpha$  secretion, because hepatocytes do not express TLR9 and viral replication was not suppressed in mice genetically deficient in the type I IFN receptor. TLR9 activation leads to improved survival when given four days post-infection in a Friend leukaemia virus model<sup>76</sup>, and even when given more than 1 week after an indolent *Leishmania major* challenge<sup>62</sup>. Protective effects of CpG against *Leishmania* infectious challenge are not limited to rodents, but have also been observed in rhesus macaques, which were protected

#### Adoptive transfer

An experimental method in which lymphocytes from an antigen-primed donor mouse are introduced into an unprimed recipient mouse.

**A-class ODN 2216**



- B-class ODN PF-3512676



- C-class ODN 2395**



- Three major classes of CpG ODN that are structurally and phenotypically distinct have been described. Examples of each class are shown in the figure, using the ID numbers from the published reports (PF-3512676 formerly was also known as ODN 2006 and CPG 7909), together with the immune effects and structural characteristics that are specific to the class. The A-class CpG ODN (also referred to as type D) are potent inducers of interferon- $\alpha$  (IFN $\alpha$ ) secretion (from plasmacytoid dendritic cells), but only weakly stimulate B cells. The structures of A-class ODN include poly-G motifs (three or more consecutive guanines) at the 5' and/or 3' ends that are capable of forming very stable but complex higher-ordered structures known as G-tetrads, and a central phosphodiester region containing one or more CpG motifs in a self-complementary palindrome. These motifs cause A-class ODN to self-assemble into nanoparticles.<sup>193</sup> B-class ODN (also referred to as type K) have a completely phosphorothioate backbone, do not typically form higher-ordered structures, and are strong B-cell stimulators but weaker inducers of IFN $\alpha$  secretion.<sup>10</sup> However, if B-class CpG ODN are artificially forced into higher-ordered structures on beads or microparticles, in dendrimers or with cationic lipid transfection, they exert the same immune profile as the A-class CpG ODN, thereby linking the formation of higher-ordered structures to biological activity.<sup>15,193-195</sup> The C-class CpG ODN have immune properties intermediate between the A and B classes, inducing both B-cell activation and IFN $\alpha$  secretion.<sup>43,196,197</sup> These properties seem to result from the unique structure of these ODN, with one or more 5' CpG motifs, and a 3' palindrome, which is thought to allow duplex formation within the endosomal environment.

There are few reported experimental models in which pretreatment with CpG exacerbates infection. However, the immune expansion induced by TLR9 activation in rodents increases the number of susceptible target cells for Friend leukaemia virus, resulting in a more aggressive infection following challenge several days later<sup>86</sup>, and CpG priming shortened survival slightly in a *Candida albicans* challenge model, in which T<sub>H</sub>1 cytokines are detrimental<sup>87</sup>. In addition, TLR9 activation with bacterial

## REVIEWS

## Box 4 | Characteristics of CpG oligodeoxynucleotides

## Drug-like characteristics

- Excellent aqueous solubility.
- Spontaneous intracellular uptake, by certain immune cells (including especially those that express Toll-like receptor 9 (TLR9)).
- Relatively simple solid-phase Good Manufacturing Practice synthesis (multi-kilogram scale) and chromatographic purification.
- Comparatively well-understood chemistry enables diverse studies of structure-activity relationships.
- Metabolites are mostly normal components of DNA, not novel small molecules.
- Range of backbones available for modulating compound stability for different applications.
- Can be administered through virtually any drug route (including oral).
- Dose exposure required for immune stimulation is ~0.1–1% of that required for antisense applications.
- Excellent stability in aqueous solutions at physiologic pH, even at room temperature.
- Well-developed highly analytic methods for Chemistry, Manufacturing and Controls (liquid chromatography–mass spectrometry is state of the art).
- Very sensitive methods available for detection of 'cold' compound<sup>201</sup>.

## Non-drug-like characteristics

- Medium size: molecular mass ~6,000–8,000 Da (length typically 18–25 bases).
- Highly charged polyanions.
- Phosphorothioate and some other backbones are chiral.
- Poor stability of purines in acid solution.
- Cleaved by nucleases in serum or cell extracts (phosphorothioate backbone is relatively nuclease resistant).
- Highly protein bound.
- Non-uniform organ distribution: highest tissue levels in kidney, liver and spleen after systemic delivery.
- Pharmacokinetics do not match pharmacodynamics after subcutaneous delivery.
- Sequence-independent effects, including concentration-dependent activation of complement proteins and prolongation of partial thrombin time.

DNA can induce the HIV transcriptional regulatory elements in long terminal repeats (LTRs)<sup>88</sup>, increasing viral replication. HIV-infected humans treated with a B-class ODN that contained a CpG motif showed dose-dependent increases in plasma HIV branched DNA levels, which represent the level of virus in the blood<sup>89</sup>. Because of the possibility of inducing increased HIV expression, CpG ODN therapy of HIV-infected individuals should probably only be undertaken during HAART (highly active antiretroviral treatment), unless the therapy is part of a clinical trial strategy to induce anti-HIV immunity. Despite their capacity to induce HIV transcription, CpG ODN can also show anti-HIV activity: the high level of IFN $\alpha$  production induced by A-class ODN suppresses HIV replication in human foetal thymus cells<sup>90</sup>, and B-class ODN can also suppress HIV replication in cultured human cells, albeit in a sequence-independent fashion<sup>91</sup>. HIV-infected long-term non-progressors have much stronger natural killer cell activation in response to A-class CpG ODN compared with progressors<sup>92</sup>, but it is not clear whether this difference in CpG-responsiveness is a cause or a consequence of the patient's clinical status. As will be discussed further below, a B-class CpG ODN has been used as a vaccine adjuvant in HIV-infected

humans on HAART with no apparent increase in HIV expression<sup>93</sup>, providing support for the cautious application of TLR9-based immunotherapeutic approaches.

**Enhancing vaccines with TLR9 agonists.** TLR9 activation enhances antigen-specific humoral and cellular responses to a wide variety of antigens, including peptide or protein antigens, live or killed viruses, dendritic cell vaccines, autologous cellular vaccines and polysaccharide conjugates in both prophylactic and therapeutic vaccines in numerous animal models. The mechanisms that contribute to the strong adjuvant activity of CpG ODN potentially include synergy between TLR9 and the B-cell receptor, which preferentially stimulates antigen-specific B cells<sup>16</sup>; inhibition of B-cell apoptosis<sup>35</sup>; enhanced immunoglobulin G (IgG) class switch DNA recombination<sup>94–96</sup>; and dendritic cell maturation and differentiation, resulting in enhanced activation of T<sub>H</sub>1 cells and strong cytotoxic T-lymphocyte (CTL) generation, even in the absence of CD4 T-cell help<sup>97,98</sup>. Conjugation of a CpG ODN directly to an antigen can enhance antigen uptake and reduce antigen requirements<sup>99,100</sup>, but cysteine residues in peptides or proteins can also form spontaneous disulphide bonds with the phosphorothioate linkage in ODN, resulting in enhanced CTL responses without the difficulties of a separate conjugation step<sup>101</sup>.

Comparisons of different adjuvants in mouse models have demonstrated CpG ODN to be unsurpassed at inducing T<sub>H</sub>1-type responses<sup>102–105</sup>. The T<sub>H</sub>1 bias induced by TLR9 stimulation is maintained even in the presence of vaccine adjuvants such as alum or incomplete Freund's adjuvant (IFA) that normally promote a T<sub>H</sub>2 bias<sup>94,106,107</sup>. Likewise, CpG ODN can overcome the T<sub>H</sub>2 bias associated with a respiratory syncytial virus vaccine<sup>108</sup>, and with vaccination in both very young and elderly mice<sup>109–116</sup>. CpG ODN show even greater adjuvant activity when formulated or co-administered with other adjuvants or in formulations such as microparticles, nanoparticles, lipid emulsions or similar formulations, which are especially necessary for inducing a strong response when the antigen is relatively weak<sup>117</sup>. CpG ODN are also effective mucosal vaccine adjuvants for respiratory tract<sup>118–121</sup>, vaginal mucosal<sup>122</sup>, oral or intrarectal vaccination<sup>121,123–125</sup>, conjunctival vaccination<sup>126</sup> and even for transcutaneous immunization<sup>127</sup>. Vaccination through mucosal routes has succeeded in inducing both local and systemic humoral and cellular immune responses, including enhanced protection against infectious challenge<sup>119,128</sup>.

In humans, CpG ODN have been used as adjuvants for hepatitis B vaccination either in combination with alum<sup>129</sup> or alone<sup>130</sup>. In a randomized, double-blind controlled Phase I/II dose-escalation study, healthy individuals received three intramuscular injections (using the FDA-approved vaccination regimen of 0, 4 and 24 weeks) of an alum-absorbed HBV vaccine either in saline or mixed with a B-class ODN, CPG 7909, at doses of 0.125, 0.5 or 1.0 mg<sup>129</sup>. Hepatitis B surface antigen (HBsAg)-specific antibody responses (anti-HBs) appeared earlier and had higher titres at all time points from 2 weeks after the initial prime up to 48 weeks in CPG 7909 recipients compared with those individuals who received vaccine

## Adjuvant

An agent mixed with an antigen that enhances the immune response to that antigen upon immunization.



alone. Moreover, most of the subjects who received CPG 7909 as adjuvant developed protective levels of anti-HBs IgG within just 2 weeks of the priming vaccine dose, compared with none of the subjects receiving the commercial vaccine alone<sup>129</sup>. In this study, the addition of the TLR9 agonist also increased the proportion of antigen-specific high-avidity antibodies, suggesting enhancement of the late-affinity maturation process in the activated B cells<sup>131</sup>.

The capacity of CPG 7909 to accelerate seroconversion was also demonstrated when it was used as an adjuvant to the approved anthrax vaccine in a randomized controlled trial in healthy volunteers. Control subjects reached their peak titre of toxin-neutralizing antibody at day 46, but this titre was already achieved in the subjects receiving CPG 7909 at day 22, more than 3 weeks earlier<sup>132</sup>. More rapid seroconversion to the anthrax toxin could be of great importance in the setting of a bioterrorist attack. Furthermore, the addition of CPG 7909 induced a statistically significant 8.8-fold increase in the peak titre of toxin-neutralizing antibody, and increased the proportion of subjects who achieved a strong IgG response to the anthrax protective antigen from 61% to 100%<sup>132</sup>. These results indicate great potential for TLR9 agonists as vaccine adjuvants in both mice and humans, despite the differences in immune cells expressing TLR9 between these species.

Certain populations are hyporesponsive to vaccination, especially immune-suppressed individuals such as those infected with HIV. A randomized double-blind controlled trial in HIV-infected humans who previously had failed to respond to an HBV vaccine, Engerix-B, alone demonstrated that addition of CPG 7909 to the vaccine significantly enhanced both the mean titres of anti-HBs and the antigen-specific T-cell proliferative response<sup>93</sup>. Perhaps of equal import, the proportion of HIV patients who had seroprotective levels at 12 months following vaccination was increased from 63% in the controls to 100% in the group receiving CPG 7909<sup>93</sup>. Moreover, with CPG 7909 the protective antibody levels and the significantly enhanced antigen-specific lymphocyte proliferation were maintained for more than a year<sup>93</sup>.

The use of CpG ODN as a vaccine adjuvant in mice enables the antigen doses to be reduced by approximately two orders of magnitude, with comparable antibody responses to the full-dose vaccine without CpG<sup>133</sup>. In a Phase Ib randomized, double-blind controlled clinical trial, subjects vaccinated with a one-tenth dose of a commercial trivalent killed split influenza vaccine (Fluarix; GlaxoSmithKline) had reduced levels of antigen-specific IFN $\gamma$  secretion from re-stimulated peripheral blood mononuclear cells (PBMC) compared with those measured in PBMC from subjects vaccinated with the full-dose vaccine alone<sup>134</sup>. However, the co-administration of CPG 7909 with the one-tenth dose of Fluarix restored the antigen-specific IFN $\gamma$  secretion to the level seen with full-dose vaccine<sup>134</sup>.

The T<sub>H</sub>1-biased immune effect of CpG ODN has been applied to the development of allergy vaccines, which in mice are able to redirect the allergic T<sub>H</sub>2 response and

prevent inflammatory disease manifestations, even in mice with established allergic disease<sup>135,136</sup>. A conjugate of a CpG ODN to a portion of the ragweed allergen has been evaluated as an allergy vaccine in human clinical trials, which provided encouraging evidence for a selective and specific redirection of the allergic T<sub>H</sub>2 response towards a non-allergic and non-inflammatory T<sub>H</sub>1 response, and a significant clinical benefit with reduced allergic symptoms<sup>137,138</sup>.

In a small Phase I tumour vaccine trial using a 1-mg dose of CPG 7909 as adjuvant to recombinant melanoma antigen family A, 3 (MAGEA3) tumour antigen for tri-weekly vaccination in six patients with metastatic melanoma, there were two stable disease and two partial responses beginning after seven to ten vaccinations, and lasting at least a year as assessed by RECIST (Response Evaluation Criteria In Solid Tumors)<sup>139</sup>. In eight melanoma patients, CPG 7909 at a dose of 0.5 mg stimulated strong and rapid CD8 T-cell responses to a Melan-A tumour peptide antigen when used with Montanide (Seppic) as a cancer vaccine adjuvant<sup>140</sup>. Taken together, the results from these human clinical trials show that stimulation of TLR9-expressing cells (presumably pDC and B cells) is sufficient to induce strong and sustained humoral and cellular memory immune responses, even in those with HIV infection, allergy or cancer, offering several advantages over conventional vaccines (TABLE 2, BOX 5).

**Directing adaptive immunity without a vaccine.** Historically, induction of effective antigen-specific immune responses has required a vaccine. However, there are several therapeutic fields in which TLR9 activation has been applied to achieve a similar effect, but without a vaccine. For example, although allergy vaccines with CpG ODN typically provide rapid redirection of allergic responses, inhaled CpG ODN monotherapy given repeatedly can prevent or treat allergic airway responses not only in mouse models<sup>141</sup> but also in primates<sup>142</sup>. Potential mechanisms that have been proposed to explain the somewhat counterintuitive anti-inflammatory effect of TLR9 stimulation on pulmonary inflammation include the induction of a T<sub>H</sub>1-like cytokine milieu that suppresses the T<sub>H</sub>2 response, systemic expression of IL-10 or transforming growth factor- $\beta$  (TGF $\beta$ ), and pulmonary expression of indoleamine (2,3)-dioxygenase (IDO)<sup>143,144</sup> (TABLES 1, 2).

CpG ODN have antitumour activity in many mouse models (reviewed in REF. 145). In relatively small tumours CpG monotherapy can be sufficient to induce a T-cell-mediated rejection of established tumours; however, to induce rejection of larger tumours the CpG ODN often needs to be combined with other effective antitumour strategies, such as monoclonal antibodies, radiation therapy, surgery and chemotherapy. Encouraging evidence for the capacity of TLR9 activation to induce a T<sub>H</sub>1-like cytokine response in human cancer patients has been reported recently in studies of dendritic cells isolated from primary human tumours<sup>100</sup> and in lymphoma patients treated with a CpG ODN alone or together with an antitumour antibody<sup>146,147</sup>. Chemotherapy has historically been considered to be immune suppressive, so it

**Seroconversion**  
Development of a detectable concentration of pathogen-specific antibodies in the serum as a result of infection or immunization.

**Table 2 | Therapeutic applications for TLR9 agonists**

| Therapeutic approach      | Animal models   | Human clinical trials   | Proposed mechanism of action  |
|---------------------------|---|---|---|
| <b>Infectious disease</b> |   |   |   |
| Monotherapy               | Many, especially against viruses and intracellular bacteria; reviewed in REF. 200   | • C-class ODN CPG 10101 <sup>85</sup> in Phase II (Coley) for hepatitis B   | Innate immune activation, with T <sub>H</sub> 1-like cellular and cytokine/chemokine responses  |
| Vaccines                  | Many, reviewed in REF. 202  | • B-class ODN 1018 ISS (Phase III; Dynavax) and CPG 7909 (Phase I; GlaxoSmithKline (GSK)/Coley and DARPA/NIAID/Coley) for hepatitis B <sup>93,129,130,203</sup> influenza <sup>134</sup> , anthrax <sup>132</sup> and other indications   | Enhancing antigen-specific humoral and cellular adaptive immune responses   |
| <b>Cancer</b>             |   |   |   |
| Monotherapy               | Many, (especially intratumoral injection) reviewed in REF. 145  | • B-class ODN PF-3512676 <sup>146</sup> (Phase I; Pfizer/Coley)   | NK-cell mediated in B16IP melanoma model, T-cell mediated in most other models  |
| Vaccines                  | Many, including peptide or protein antigens, carbohydrate conjugates, whole cell vaccines and DC vaccines, reviewed in REF. 145                       | • B-class ODN PF-3512676 (Phase I; Pfizer/Coley and GSK/Coley) with Melan-A peptide <sup>140</sup> , and with MAGE recombinant protein <sup>139</sup>   | CD4 and/or CD8 T-cell mediated  |
| Combination therapies     | Various, including chemotherapy, radiotherapy, surgery, immunotherapy; reviewed in REF. 145   | • B-class ODN 1018 ISS + Rituximab for NHL <sup>147</sup> (Phase I; Dynavax)<br>• PF-3512676 combined with taxane/platin chemotherapy for NSCLC <sup>153</sup> (Phase III; Pfizer/Coley)<br>• HYB-2055 in combination with gemcitabine and carboplatin for refractory solid tumours (Phase II; Idera) | TLR9 stimulation enhances ADCC for combination with mAb; chemotherapy seems to preferentially reduce regulatory T-cell function, enhancing the CpG-induced antitumour T-cell response                         |
| <b>Asthma/allergy</b>     |   |   |   |
| Monotherapy               | Mouse: asthma, allergic rhinitis, conjunctivitis, allergic aspergillosis<br>Guinea pig: RSV sensitization<br>Monkey: asthma. All reviewed in REF. 143 | • AVE 7279 (Phase I; sanofi-aventis/Coley)<br>• AVE 0675 (preclinical; sanofi-aventis/Coley)<br>• 1018 ISS (Phase II; Dynavax)<br>• IMO (preclinical; Novartis/Idera)   | Suppress T <sub>H</sub> 2 response and IgE production <sup>204</sup> . Induce IDO expression, promoting anti-inflammatory Treg cells and reverse airway remodelling <sup>144</sup> . All reviewed in REF. 143 |
| Vaccines                  | Mouse: asthma, allergy<br>Immunotherapy and atopic dermatitis   | • B-class ODN 1018 ISS conjugated to protein <sup>137,138</sup> (Phase III; Dynavax)  | Suppress or redirect T <sub>H</sub> 2 allergic response   |

ADCC, antibody dependent cellular cytotoxicity; DARPA, Defense Advanced Research Projects Agency; DC, dendritic cell; IDO, indoleamine (2,3)-dioxygenase; IP, intraperitoneally; mAb, monoclonal antibody; NHL, non-Hodgkins lymphoma; NIAID, National Institute of Allergy and Infectious Disease; NK, natural killer; NSCLC, non-small-cell lung cancer; ODN, oligodeoxynucleotides; RSV, respiratory syncytial virus.

might seem counterintuitive to combine this with TLR9 stimulation, and surprising that such combinations result in substantial improvements in survival in mouse tumour models using chemotherapy regimens ranging from the topoisomerase I inhibitor topotecan (Hycamtin; GlaxoSmithKline) to the alkylating agent cyclophosphamide and the antimetabolite 5-fluorouracil<sup>148–150</sup>. Where it has been tested, the increased antitumour efficacy of these combination approaches requires T cells but not natural killer cells, which is consistent with the hypothesis that *in vivo* activation of dendritic cells through TLR9 promotes an antitumour T-cell response that is capable of controlling the tumour and improving survival (FIG. 1).

Humans receiving certain chemotherapy regimens, such as taxanes, actually show increased T-cell and natural killer-cell immune competence<sup>151</sup>, which might be related to induction of proinflammatory cytokine production, induction of homeostatic leukocyte proliferation, and reversal of the immune suppressive effects of regulatory T cells (Treg cells), which seem to protect the tumour against immune rejection<sup>152</sup>.

On the basis of positive results in mouse tumour models, the effects of adding the B-class CpG ODN PF-3512676 (formerly called CPG 7909) to standard taxane/platinum chemotherapy for first-line treatment of stage IIIb/IV non-small-cell lung cancer (NSCLC) were investigated. In a Phase II randomized controlled human clinical trial, 112 chemotherapy-naïve patients were randomized to receive four to six three-week cycles of standard chemotherapy alone or in combination with 0.2 mg per kg subcutaneous PF-3512676 on weeks two and three of each cycle. The primary endpoint for the trial — response rate (assessed by RECIST, using intention-to-treat analysis) — was significantly improved from 19% in the patients randomized to standard chemotherapy to 37% in the patients who also received PF-3512676<sup>153</sup>. The secondary endpoint of this trial, survival, showed a trend towards improvement from a median survival of 6.8 months in the chemotherapy arm to 12.8 months in the combination arm and an improvement in the 1-year survival from 33% to 50%<sup>153</sup>. As in the other clinical trials with TLR9 agonists, the most common side effects were



**Box 5 | Enhancing vaccines with TLR9 agonists**

A number of shortcomings of current vaccines have been enhanced by the addition of a Toll-like receptor 9 (TLR9) agonist in human trials or preclinical mouse models.

**Vaccine deficiencies**

- Need for several boosts to achieve protection
- Delay in rise of protective antibody titres
- Prevalence of vaccine non-responders, especially among immune-compromised populations
- Cost of antigen production
- Poorly protective antibody with low avidity
- Fall in antibody titre over time

**Effect of TLR9 agonist**

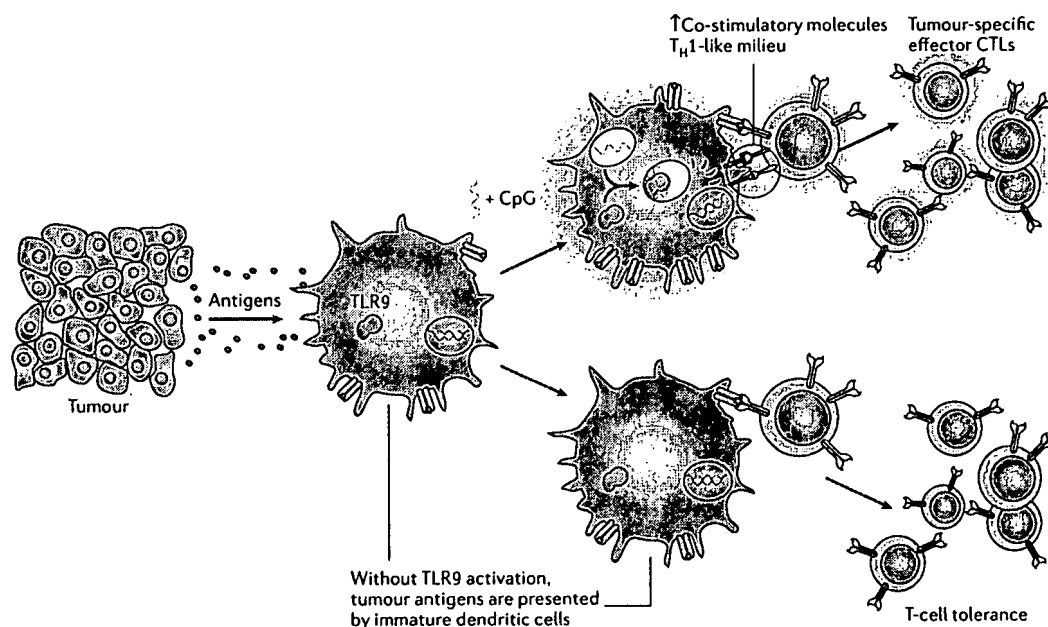
- Reduce number of vaccinations required to achieve seroprotection
- Accelerate seroconversion, possibly permitting post-exposure vaccination
- Reduce non-responder rate
- Reduce amount of antigen required
- Increase antibody avidity and protective activity
- More sustained antibody levels

mild to moderate injection-site reactions and transient flu-like symptoms. Grade 3 or 4 neutropaenia was more common in the combination arm, which is thought to reflect neutrophil redistribution, but febrile neutropaenia and grade 3/4 infections were actually slightly less common in the combination arm than in the chemotherapy alone arm. Thrombocytopaenia, a previously recognized phosphorothioate backbone effect that has occurred in all trials of antisense ODN, was seen more commonly

in the combination arm, but there was no apparent increase in bleeding events. Based on these encouraging results, two controlled Phase III human clinical trials of PF-3512676 combined with doublet chemotherapy in first-line treatment of unresectable NSCLC were initiated by Pfizer in late 2005.

**Safety of TLR9 activation in rodents and humans**

In addition to the mechanism of action-related immune effects resulting from TLR9 activation, PS-ODN have a variety of sequence-independent backbone-related effects that have been characterized in detailed studies of antisense ODN<sup>58,154,155</sup>. PS-ODN are rapidly cleared from the circulation into the liver, kidneys and, to a lesser extent, the spleen and bone marrow<sup>156,157</sup>. Chronic dosing of PS-ODN in rodents results in a dose-dependent mononuclear cell infiltration in these organs, but such changes do not occur in monkeys or humans<sup>58,158</sup>. Hepatic effects specific to rodents include the activation of Kupffer cells with cellular hypertrophy and hyperplasia, basophilic granulation (thought to reflect PS-ODN deposition), and a mononuclear cell infiltrate in hepatic sinusoids and periportal regions<sup>58,159</sup>. In the kidneys, high local ODN concentrations reached after repeated high doses can induce degenerative lesions and necrosis in proximal tubules<sup>58,160</sup>. There have been no reports of adverse effects of PS-ODN on renal function in humans, despite the extensive clinical experience so far. Presumably these species-specific toxicities are a consequence of the cellular pattern of TLR9 expression, which determines the cytokines that will be produced in response to administration of a CpG ODN, and therefore the safety profile



**Figure 1 | Switching on antitumour immunity by in vivo dendritic cell activation through TLR9.** In general, malignant tumours suppress immune function, and create an environment that favours the maintenance of T-cell tolerance, preventing the development of antitumour immunity. In vivo dendritic cell activation through Toll-like receptor 9 (TLR9) creates a  $T_H1$ -like cytokine and chemokine milieu and can up-regulate the expression of co-stimulatory molecules on the plasmacytoid dendritic cell (pDC), shifting T cells from tolerance, to a strong cytotoxic T-lymphocyte response against the tumour antigens.

of the drug. As TLR9 is expressed in a broader range of immune cells in rodents compared with primates, the rodent tends to over-predict toxicities that will occur in primates. For example, rodents respond to CpG ODN administration with high serum concentrations of pro-inflammatory cytokines such as TNF $\alpha$ , which can result in a lethal 'cytokine storm'<sup>164</sup>, but in humans and primates there is no change in serum TNF $\alpha$  following CpG injection, which is generally well tolerated<sup>59</sup>.

The major dose-limiting acute toxicity of PS-ODN in primates results from systemic activation of the alternative complement pathway with activation of leukocytes and changes in vascular permeability that can culminate in lethal cardiovascular collapse<sup>162,163</sup>. Fortunately, this toxicity does not occur below a threshold PS-ODN blood concentration of approximately 40–50  $\mu$ g per ml, which typically is only reached when ODN is given via relatively rapid intravenous administration<sup>58,162,164</sup>. Inhibition of coagulation has been reported to result from binding of the PS-ODN to thrombin (specifically, to the tenase complex), and is reflected by prolongation of the activated partial thromboplastin time<sup>58,165,166</sup>.

TLR9 activation by CpG ODN could also be proposed to induce adverse effects resulting from its mechanism of action. Early hypotheses that exposure to DNA containing CpG motifs generally induce autoimmunity<sup>167</sup> have proven unfounded. Nevertheless, CpG ODN treatment can clearly exacerbate autoimmunity in mouse models of lupus<sup>168</sup>, multiple sclerosis<sup>169</sup>, colitis<sup>170</sup> and arthritis<sup>171</sup>. Evolutionary considerations would suggest that the TLR9 pathway should not have evolved as an important immune defence mechanism unless its activation was controlled in some way, so as to prevent or at least limit the risk of inducing autoimmunity. Indeed, studies in various experimental models have established that TLR9 stimulation induces its own feedback suppression through mechanisms including induction of IFN $\alpha$ <sup>172</sup> or IFN $\gamma$  secretion<sup>173,174</sup>; increased expression of IDO (which might promote development of immune-suppressive Treg cells)<sup>144,172–176</sup>, cyclooxygenase-2 (COX2)<sup>177</sup> and suppressor of cytokine signalling, (SOCS); decreased expression of IRAK; and activation of ERK. There also seem to be some constitutively active pathways operating to limit the effects of TLR activation, such as single

immunoglobulin IL-1R-related molecule (SIGIRR). The existence of these diverse counter-regulatory pathways that limit TLR9-induced immune activation suggests a potential to enhance the therapeutic efficacy of TLR9 agonists by co-administration of antagonists of one or more of these inhibitory pathways. Of course, such combinations might have a greater risk of inducing autoimmune disease. Understanding these mechanisms could make it possible to increase the therapeutic efficacy of CpG ODN by selectively disabling one or more of these counter-regulatory pathways, without inducing substantial added toxicity.

The safety profile of several TLR9 agonists in humans has been observed in the clinical trials described above over a more than 1,000-fold dose range from 0.0025–0.81 mg per kg. A maximal tolerated dose in humans has not been reported to date. The primary adverse events are dose-dependent local injection reactions (such as erythema, pain, swelling, induration, pruritus or warmth at the site of injection) or systemic flu-like reactions (such as headache, rigors, myalgia, pyrexia, nausea and vomiting), and are consistent with the known TLR9 agonist mechanism of action. Depending on the dose, systemic symptoms typically develop within 12–24 hours of dosing and persist for 1–2 days. At the low doses used in vaccine trials there seems to be a slight increase in the frequency of injection-site reactions, which are generally mild, above the frequency observed with the vaccine alone.

The clinical experience to date indicates that CpG ODN treatment of normal humans, cancer patients or individuals infected with HIV or HCV does not readily induce autoimmune disease. However, the duration of therapy has usually been less than 6 months; only a few patients have received chronic therapy with CpG ODN for longer than 3 years. In some animal models CpG ODN can even prevent autoimmune or inflammatory disease<sup>172</sup>, but from a clinical perspective it might be prudent to consider the safety effects of CpG ODN in the same category as recombinant IFN $\alpha$ . Extensive clinical experience with IFN $\alpha$  has documented the induction of an autoimmune disease in 4–19% of chronically treated patients, and systemic lupus erythematosus (SLE) has been diagnosed in 0.15–0.7% of patients<sup>178</sup>. In most cases, such diseases resolve spontaneously after drug withdrawal. Based on the clinical experience to date, it seems that the incidence of autoimmunity and the overall toxicity will be lower with CpG ODN than has been observed with IFN $\alpha$  therapy; however, no definite conclusion on this can be reached until larger numbers of patients have been treated with CpG ODN for longer periods of time.

## Future outlook

The CpG motif was described in 1995, and TLR9 was recognized to be the target of CpG DNA in 2000. Since then, half a dozen TLR9 agonists have been taken into human clinical trials, including three investigational products in Phase III trials, and there are already strong indications of substantial clinical benefit: it seems likely that the targeted activation of TLR9 using CpG ODN will enhance the treatment of cancer and infectious diseases, as well as offering new prospects for decreasing

## Box 6 | Some unanswered questions and uncertainties in TLR9 biology

- Is Toll-like receptor 9 (TLR9) expression regulated under physiological conditions, and by what stimuli?
- What cells express TLR9 in normal and disease states, and how does this explain the species-specific effects of CpG?
- What is the molecular basis for the different classes of CpG oligos?
- Does TLR9 directly and specifically bind CpG motifs?
- How does the interaction between TLR9 and the CpG motif activate signal transduction?
- Why can immune complexes containing mostly methylated vertebrate DNA activate immune cells, whereas vertebrate DNA alone does not?
- Could chronic TLR9 activation induce autoimmune disease in some people?
- What is the clinical significance of TLR9 polymorphisms, and how much heterogeneity is there in human responses to CpG ODN?

the harmful inflammatory responses that characterize asthma and other allergic diseases. The rapidity of this clinical development and the breadth of the positive clinical data are impressive compared with the usual course of drug development against a novel target. The success of TLR9-based approaches has led to a resurgence of interest in the induction of therapeutic innate and adaptive immune responses. Although more studies are needed, and important questions remain to be addressed (BOX 6), the safety of these TLR9 agonists seems good.

A new direction in targeting TLR9 is suggested by recent studies implicating inappropriate activation of

TLR9 by endogenous molecules in the pathogenesis of SLE and rheumatoid arthritis<sup>179–183</sup>. The results of these studies suggest that *antagonists* of TLR9 could be useful in the treatment of these autoimmune diseases, by blocking this inappropriate activation of B cells and pDC. Indeed, in mouse models, suppressive ODN designed to block TLR9 have already shown benefit in preventing or reversing both SLE and rheumatoid arthritis<sup>54,184,185</sup>. TLR9 could turn out to be a target for which both agonists and antagonists could find therapeutic application, depending on the clinical setting. The coming years and a lot of work should provide the answer to this question.

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## Competing interests statement

The author declares competing financial interests: see Web version for details.

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The following terms in this article are linked online to:  
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